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Glomerular cells *in vitro* versus the glomerulus *in vivo*

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Early data on glomerular physiology and pathophysiology were largely based on morphological studies and on functional studies of either glomeruli *in vivo* or isolated glomeruli. The introduction of glomerular cell culture in the late seventies and early eighties has allowed a more specific approach to the role of individual glomerular cell types in the mediation of renal pathology [1–3]. Over the last five years there has been a tremendous increase in studies dealing with cultured glomerular cells. For example, the number of abstracts on mesangial cells in culture that have been presented at the annual meeting of the American Society of Nephrology has increased from about 10 in 1988 to about 150 in 1992. In contrast, only about 25 studies dealt with glomerular epithelial cells last year, and very few with glomerular endothelial cells, which is likely a reflection of the ease at which these cells can be grown out of isolated glomeruli. As discussed in several recent papers, these *in vitro* studies have shown that glomerular cells have a wide range of synthetic activities and response patterns [this issue of *Kidney International*, 1–8].

Based on the *in vitro* characterization of the capabilities of glomerular cells, physiological or pathological pathways have been suggested that may be operative *in vivo*. However, when working with cells isolated from glomeruli and when attempting to extrapolate to the *in vivo* situation, several issues need to be addressed. First, what type of cells has been obtained and how do their morphological characteristics compare to those observed *in vivo*? Second, is there evidence to suggest that cultured cells exhibit the same synthetic profile as they do *in vivo* or do they synthesize products that can not be detected *in vivo* under normal or pathological circumstances? Third, are the responses of cultured glomerular cells comparable to those observed *in vivo* or do they show response patterns that are restricted to the petri dish?

In the following review we will discuss these three issues by way of example rather than by attempting to give a complete overview. Finally, we will restrict our discussion to glomerular mesangial and epithelial cells, since too little information is available on glomerular endothelial cells either in culture or *in vivo*.

Cells in culture, but which ones?

Glomerular cells have been derived from either intact, isolated glomeruli (epithelial cells) or collagenase digested isolated glomeruli (mesangial cells). For this, glomeruli are placed into tissue culture medium, containing high concentrations of nutrients and growth factors, and are then regularly checked for cellular outgrowths [1–3]. Until recently (see below) such cultures were performed in two dimensional culture on either uncoated petridishes (mesangial cells) or petridishes that were pretreated with various attachment factors, usually some extracellular matrix protein (epithelial cells) [1–3]. The isolation of individual glomerular cell types from these primary outgrowths was then achieved by either cloning methods (rarely, since not very efficient) or, more frequently, by repeated subculture under conditions that select for one of the cell types [1–3]. Cells are then characterized using morphological, immunohistological and functional criteria [1–3].

The approach described above has some specific inherent problems that may complicate the isolation of individual cell types. First, the preparation of glomeruli from whole kidney or renal cortex almost never yields an absolutely pure population of glomeruli, but is usually contaminated to low degrees with tubular and presumably interstitial tissue. Second, although most glomeruli lose their capsule during the isolation procedure, Bowman's capsule, various lengths of the proximal tubule, and/or the vascular pole may still be attached to some isolated glomeruli. Although morphologic, immunohistochemical, and functional methods may distinguish many of the cell types from each other, this is not the case in other instances. For example, it may be difficult to exclude the possibility that mesangial cell preparations are contaminated by either interstitial fibroblasts or smooth muscle cells, both of which show a number of features reminiscent of mesangial cells [4, 6, 9], or to differentiate whether a preparation of glomerular epithelial cells contains predominantly visceral or parietal glomerular epithelial cells [10–12].

Other caveats originate from the cell culture conditions themselves. As outlined by Lovett and Sterzel in 1986 [3], glomerular cell characteristics will depend on species, age of donor, and the degree of confluency of the cells. Also, the matrix, no matter whether synthesized by the cells themselves or provided in the petri dish, may exert marked influences on the morphology and behavior of the cultured cells [13, 14]. Another, very important variable is the time that a cell has been in culture. Thus, with increasing numbers of passages the risk of selecting subpopulations or even contaminating cell types is likely to increase and chromosomal alterations become very frequent [15] (Fig. 1). This latter point is of particular concern

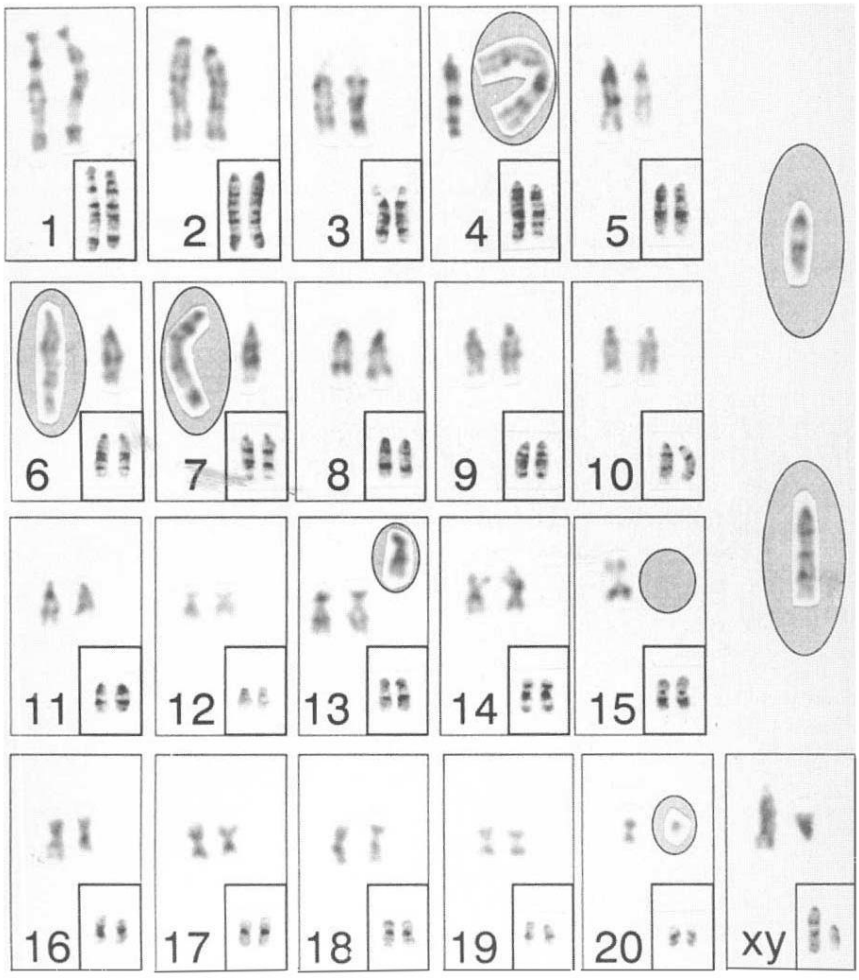

Fig. 1. *Comparison of the normal chromosomal set of rats (*Rattus norvegicus*) (small insert boxes) with a representative karyotype of cultured Sprague-Dawley rat mesangial cells that could be maintained in culture for more than 20 passages.* The karyogram of the cells after prolonged culture shows several structural and numerical aberrations (grey oval circles): One chromosome 4 is replaced by a Robertsonian translocation t(4;4), one of chromosomes number 6 and 7 has additional parts attached to the long arms, there is a third chromosome 13, one chromosome 15 is deleted, one chromosome 20 shows a partial deletion, and finally two marker chromosomes (non-classifiable chromosomes) are present.

when immortalized glomerular cells, for example virus-transformed cells, are investigated. With most glomerular cells in culture, however, a high number of passages is not possible since they will spontaneously cease to proliferate after a certain time in culture. If this is not the case, either a dedifferentiation or a spontaneous virus-transformation must be assumed. A last caveat is derived from the repeated usage of non-autologous serum to propagate cells, which contains not only platelet release products and products of the coagulation pathway but frequently considerable amounts of endotoxin. Thus, it is clear from this discussion that the interpretation and particularly the comparison of cell culture data should only be attempted after a thorough analysis of the conditions under which the cells were obtained.

Synthetic activities in culture and *in vivo*

As outlined in the previous articles, both glomerular mesangial and epithelial cells in culture are metabolically active cells. Major classes of synthesized products include inflammatory mediators, structural proteins, such as elements of the cytoskeleton, and extracellular matrix proteins. In the following we will compare the synthesis of selected proteins out of these three classes *in vitro* with that observed under normal or pathological conditions *in vivo*.

Mesangial cells

Inflammatory mediators

As an example, we will analyze the synthesis of platelet-derived growth factor (PDGF) *in vitro* and *in vivo*. PDGF is a mediator that, among other biological actions, is involved in organogenesis, wound healing, and the pathogenesis of atherosclerosis, fibrotic diseases and inflammatory joint disease [16]. In cultured mesangial cells, PDGF synthesis can be demonstrated [17] and PDGF exhibits mitogenic (see below) [18] and chemotactic [19] activities for these cells. It is also involved to some extent in the regulation of their matrix synthesis [20] as well as the synthesis of other mediators, such as prostaglandins [21]. *In vivo* PDGF B-chain mRNA and protein can be demonstrated in low to moderate amounts in the normal glomerulus, where it localizes by *in situ* hybridization and immunostaining, respectively, to mesangial regions [22–25]. In diseases that are

Table 1. Comparison of selected synthetic activities of glomerular mesangial cells in *in vitro*, under normal *in vivo* conditions, and during glomerular disease *in vivo*

	In vitro	*In vivo*	
		normal	Pathol.
Mediators			
PDGF B-chain	++ [17]	+ [22–25]	++ [22–27]
Structural proteins			
α-Smooth muscle actin	++ [28]	− [29](Rat)	++ [27, 29–31]
Desmin	++ [32]	+ [29, 33–36]	++ [29, 35, 36]
Matrix proteins			
Type I collagen	++ [45]	− [41–44]	++ [41–43, 46, 47]
Type IV collagen	+ [45]	+ [37–40, 42, 43]	++ [37–40, 42, 43]

Signs signify: −, absent; +, weak to moderate; and ++, strong expression. Numbers in brackets refer to the corresponding references.

characterized by enhanced mesangial cell proliferation, upregulated synthesis of glomerular PDGF B-chain can be demonstrated [22–27]. Thus, in the case of PDGF there is high consistency of *in vitro* and *in vivo* observations (Table 1).

Structural proteins

As shown in Table 1 and Figure 2, expression of α-smooth muscle actin can be readily demonstrated as part of the cytoskeleton of cultured rat mesangial cells [28]. In the normal rat kidney, however, α-smooth muscle actin is not present within the glomerulus (Fig. 2) [29]. An apparent *de novo* expression of α-smooth muscle actin in the mesangium *in vivo* only occurs in instances of severely altered glomerular hemodynamics, in which the mesangial cell is exposed to increased stretch: selective disruption of the mesangial architecture following administration of an anti-mesangial cell antibody (anti-Thy 1.1; Fig. 2) [29], increased glomerular capillary pressure following 5/6 nephrectomy [27] or infusion of angiotensin II [30], or hyperfiltration in diabetic nephropathy [31] (Table 1). In contrast to α-smooth muscle actin, other cytoskeletal elements of the mesangial cell, such as desmin, show a more consistent expression (Fig. 3). Thus, desmin can be demonstrated *in vitro* as well as in the normal mesangium *in vivo* and finally, in increased amounts, in the pathologically altered mesangium [29, 32–36] (Table 1).

Extracellular matrix proteins

Table 1 shows that, in contrast to type IV collagen, the normal glomerular extracellular matrix by immunostaining is devoid of interstitial types of collagen (types I and III) [37–43]. It has also been impossible to demonstrate any type I collagen mRNA in normal glomeruli even when using isolated single glomeruli and the polymerase chain reaction [44]. On the other hand, *in vitro* 95% of the collagen synthesized by mesangial cells consists of the interstitial collagen types I and III, and only very low amounts of type IV collagen are produced [45]. Under pathological conditions *in vivo* the glomerular content of type IV collagen increases [37–40, 42, 43] but the appearance of interstitial collagens in the glomerulus has also been repeatedly shown [41–43, 46, 47]. However, it has been argued that these latter collagens were derived from interstitial fibroblasts invading the glomerular tuft following the disruption of Bowman's capsule [47]. It was therefore of great interest to us to observe the ubiquitous glomerular expression of type I collagen in the anti-Thy 1.1 mesangioproliferative glomerulonephritis model in the rat [42], since in this model the injury is directed against the mesangial cells and since the disease is not characterized by breaks in Bowman's capsule [29]. This suggested that intrinsic glomerular cells, most likely the mesangial cells, were responsible for the overproduction of type I collagen in this nephritis model [42]. Thus, this model provides an *in vivo* correlate for the predominant interstitial type collagen production by cultured mesangial cells (Table 1).

Glomerular epithelial cells

Inflammatory mediators

We have recently obtained evidence showing that nonstimulated glomerular epithelial cells in culture, similar to mesangial cells, synthesize low amounts of PDGF B-chain [48]. *In vivo*, neither PDGF B-chain protein nor mRNA could be demonstrated in visceral or parietal epithelial cells of normal rat kidney by immunohistology and *in situ* hybridization, respectively [22–27]. However, following the induction of an experimental membranous nephropathy (passive Heymann nephritis), both visceral and parietal glomerular epithelial cells transiently expressed PDGF B-chain protein and mRNA [48] (Table 2).

Structural proteins

Glomerular epithelial cells in culture, like mesangial cells, are characterized by their cytoplasmic expression of vimentin, a common element of the cytoskeleton [49, 50]. Unlike mesangial cells however, they do not express desmin [51]. *In vivo*, the expression of vimentin in normal glomeruli is strong in visceral and weaker in parietal epithelial cells [33–36], while desmin may be detected in low amounts in some visceral but not in parietal epithelial cells [29, 33–36]. In contrast to vimentin, where no marked changes of the glomerular epithelial staining pattern have been observed in disease models involving the glomerular epithelial cells [35, 36], marked increases in the expression of desmin by visceral but not parietal epithelial cells can be noted when stretch is imposed on the glomerulus *in vivo* [35, 36] (Table 2). This discrepancy of the *in vitro* and *in vivo* findings in the case of visceral epithelial cells but not in parietal epithelial cells (Table 2) supports the notion that most glomerular epithelial cells in culture may in fact be of parietal rather than visceral origin [10–12].

Extracellular matrix proteins

Very similar to the mesangial cell, some type I collagen can be demonstrated in cultured glomerular epithelial cells [2, 51], while the normal glomerulus is devoid of this collagen type (see above). In experimental membranous nephropathy, however, type I collagen mRNA increases in the glomeruli [52] and by *in situ* hybridization this increase has been localized to visceral epithelial cells [53]. An apparent *de novo* expression of type I collagen protein in visceral epithelial cells has also been noted in this disease model [52]. Furthermore, expression of type I collagen in the cytoplasm of visceral as well as parietal epithelial cells was observed following 5/6-nephrectomy in rats [43]. Thus, similar to PDGF B-chain, the expression of type I collagen by glomerular epithelial cells represents an example, in

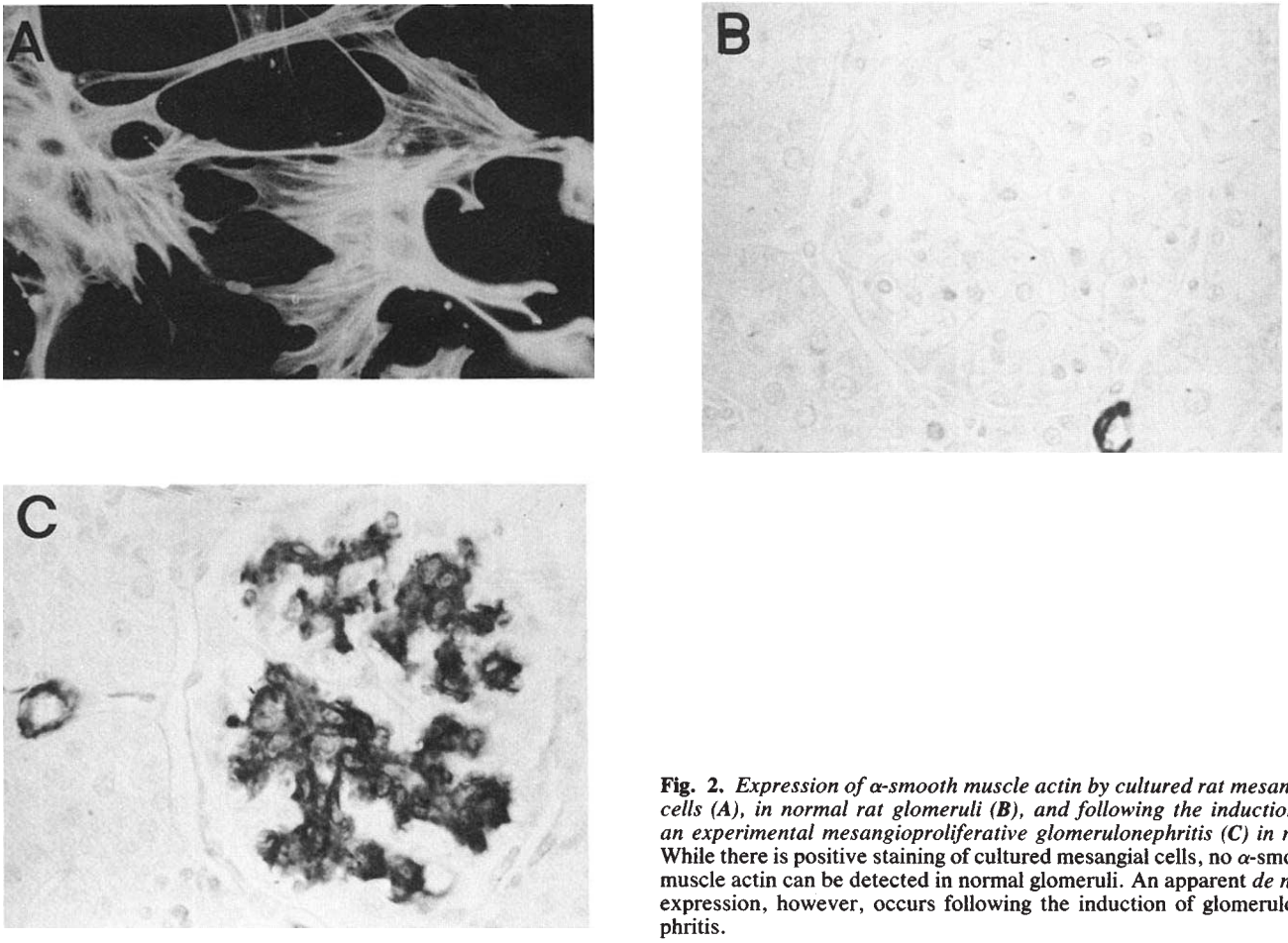

Fig. 2. *Expression of α-smooth muscle actin by cultured rat mesangial cells (A), in normal rat glomeruli (B), and following the induction of an experimental mesangioproliferative glomerulonephritis (C) in rats.* While there is positive staining of cultured mesangial cells, no α-smooth muscle actin can be detected in normal glomeruli. An apparent *de novo* expression, however, occurs following the induction of glomerulonephritis.

which the phenotype of the cultured cells resembles that of an injured cell *in vivo* rather than that of a normal cell *in vivo*.

Cellular responses in the petri dish and in the glomerulus

Mesangial cells

A major pathological response pattern of mesangial cells to injury *in vivo* is an increase in their proliferation rate. Augmented mesangial cell proliferation has been documented in a multitude of glomerular diseases, ranging from various glomerulonephritides, diabetic nephropathy to the changes following extensive loss of renal mass [27, 54–56]. Cell culture has provided an excellent opportunity to investigate the nature of the factors involved in this abnormal proliferative response. Indeed, a large number of factors with mitogenic activity for mesangial cells, some with growth inhibitory capabilities and some with no effect have been identified in such experiments [reviewed in 4, 5, 55, 57]. In contrast, however, there is relatively little information on the factors regulating mesangial cell proliferation *in vivo*.

In the normal glomerulus *in vivo* there is little cell turnover and 70 to 80% of the proliferating cells are identified as endothelial cells, while mesangial cells only account for 10 to 20% [58]. This observation is in relative contrast to data obtained in cell culture, which show that mesangial cells, at least in their early passages, are highly proliferative and that even the complete withdrawal of serum and protein from the cell culture medium can not induce a complete growth arrest of these cells [21]. In this respect cultured mesangial cells are more similar to the activated mesangial cell *in vivo*, since, as noted above, mesangial cell proliferation is a common occurrence in a variety of glomerular diseases (Table 3).

One of the most thoroughly investigated mesangial cell mitogens is PDGF and in particular the B-chain of PDGF. Thus, mesangial cells in culture exhibit a strong proliferative response when stimulated with PDGF [18, 59], and the intrinsically produced PDGF may act as an autocrine growth factor [18]. This latter observation potentially explains why it is not possible to completely growth arrest mesangial cells in culture. As noted above, *in vivo* PDGF B-chain mRNA and/or protein are expressed in low amounts in normal glomeruli and are overexpressed in mesangioproliferative states. A neutralizing antibody to PDGF was able to reduce the pathological mesangial cell proliferation observed in the anti-Thy 1.1 nephritis [60], and, vice versa, infusion of recombinant PDGF into normal rats induced selective mesangial cell proliferation *in vivo* [61]. Thus, the mesangial cell response to PDGF is another example of consistency of cell culture findings and *in vivo* observations (Table 3).

Similar to PDGF, basic fibroblast growth factor (bFGF) is also a potent mesangial cell mitogen in culture [18] and, again,

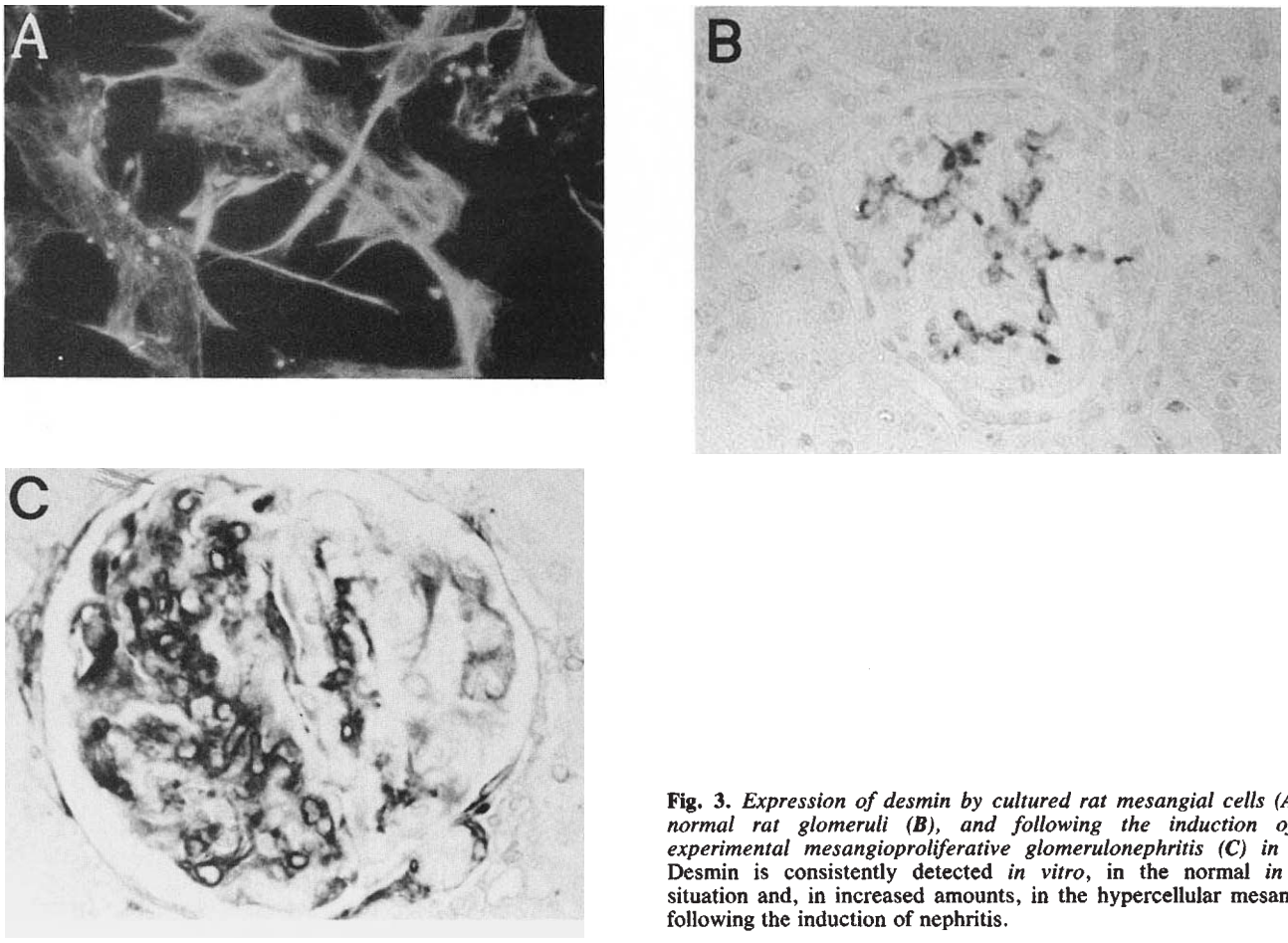

Fig. 3. *Expression of desmin by cultured rat mesangial cells (A), in normal rat glomeruli (B), and following the induction of an experimental mesangioproliferative glomerulonephritis (C) in rats.* Desmin is consistently detected *in vitro*, in the normal *in vivo* situation and, in increased amounts, in the hypercellular mesangium following the induction of nephritis.

Table 2. Comparison of selected synthetic activities of visceral ("v") and parietal ("p") glomerular epithelial cells in *in vitro*, under normal *in vivo* conditions, and during glomerular disease *in vivo*

		In vivo	
	In vitro	Normal	Pathol.
Mediators			
PDGF B-chain	+ [48]	v − [22–25] p − [22–25]	v ++ [48] p ++ [48]
Structural proteins			
Vimentin	++ [49, 50]	v ++ [33–36] p + [33, 34, 36]	v ++ [35, 36] p + [35, 36]
Desmin	− [51]	v ± [29, 33–36] p − [29, 33–36]	v ++ [35, 36] p − [35, 36]
Matrix proteins			
Type I collagen	+ [2, 51]	v − [41–44] p − [41–43]	v + [43, 52, 53] p + [43]

Signs signify: −, absent; +, weak to moderate; and ++, strong expression. Numbers in brackets refer to the corresponding references.

Table 3. Comparison of selected cellular responses of glomerular mesangial cells in *in vitro*, under normal *in vivo* conditions, and during glomerular disease *in vivo*

		In vivo	
	In vitro	Normal	Pathol.
Proliferation			
No additional stimulus	+ [1–3]	+ [58]	++ [54–56]
Proliferation following administration of:			
PDGF	++ [18, 59]	++ [61]	++ [61]
bFGF	++ [18]	− [61, 62]	+ [61, 62]
Angiotensin II	± [64–67]	− [30, 68]	− [unpubl.]

Signs indicate: −, absent; +, weak to moderate; and ++, strong response. In the case of PDGF, bFGF and angiotensin II only the increase above basal conditions (no additional stimulus) is depicted. Numbers in brackets refer to the corresponding references.

bFGF is produced by both mesangial cells in culture and mesangial cells *in vivo* [62]. However, in contrast to cell culture, neither bolus injections nor a one week infusion of recombinant bFGF into normal rats elicited a mesangioproliferative response [61, 62]. Only after the mesangial cells *in vivo* had suffered a minor injury (injection of a low dose of an anti-mesangial cell antibody), was a moderate proliferation of mesangial cells observed in response to the administration of bFGF [61, 62]. Thus, in contrast to PDGF, the cellular responsiveness of mesangial cells towards bFGF depends on their status *in vivo* and cultured mesangial cells behave similar to injured but not to normal mesangial cells *in vivo* (Table 3).

A third growth factor that has received increased attention over the last years is angiotensin II. In vascular smooth muscle cells, which in many ways resemble mesangial cells [4, 6], angiotensin II does not exert mitogenic effects but rather

Table 4. Comparison of selected cellular responses of visceral ("v") or parietal ("p") glomerular epithelial cells in *in vitro*, under normal *in vivo* conditions, and during glomerular disease *in vivo*

	In vitro	*In vivo*	
		Normal	Pathol.
Proliferation			
No additional stimulus	+ [10–12]	v − [58]	v + [48]
		p + [58]	p ++ [69, 70]
Proliferation following administration of:			
PDGF	− [48]	v − [61]	v − [61]
		p − [61]	p − [61]
bFGF	+ [73]	v − [61, 62]	v ?
		p − [61, 62]	p ?

Signs indicate: −, absent; +, weak to moderate; and ++, strong response. In the case of PDGF and bFGF only the increase above basal conditions (no additional stimulus) is depicted. Numbers in brackets refer to the corresponding references.

induces cellular hypertrophy [63]. Similarly, in cultured human mesangial cells derived from adult angiotensin II is not mitogenic [64, 65]. Reports by other authors of low grade mitogenic effects of angiotensin II may relate to the usage of fetal or immortalized mesangial cells [66, 67]. *In vivo* there was only a marginal increase in the glomerular cell proliferation rates in normal rats [30] and none in rats with a mesangioproliferative nephritis [R. Johnson, unpublished data] infused for up to two weeks with angiotensin II. More importantly, in one-clip, two-kidneys Goldblatt rats at 11 weeks after disease induction there was no effect on glomerular cell proliferation or cell counts in the clipped kidneys, which are exposed to high levels of angiotensin II but not to hypertension [68]. This cellular response towards angiotensin II therefore exemplifies that low-grade effects *in vitro* may not be demonstrable *in vivo* (Table 3), and the biological relevance of such cell culture findings has to be questioned.

Glomerular epithelial cells

If increased proliferation of glomerular epithelial cells is evaluated again as a response pattern, there is evidence that parietal epithelial cells can proliferate *in vitro* [10–12] as well as under normal and pathological conditions *in vivo* [58, 69, 70]. In the case of the visceral epithelial cells, however, it has been hypothesized that these cells are terminally differentiated *in vivo* and are therefore incapable of exhibiting any proliferative response [71]. Indeed in normal rat glomeruli, no proliferation of visceral epithelial cells has been detected [58]. We have, however, demonstrated recently that in an experimental model of membranous nephropathy (passive Heymann nephritis), in which cytotoxic injury is directed against the visceral epithelial cells, some regenerative response, that is, proliferation, of the visceral epithelial cells occurs [48]. The *in vitro* correlate of this observation is provided by the mere fact that it is possible to, at least temporarily, induce an outgrowth of not only parietal but also visceral epithelial cells from isolated glomeruli [10–12] (Table 4).

An example of consistently negative responses of glomerular epithelial cells is their response towards PDGF. Thus, both *in vitro* and following a one week infusion of recombinant PDGF *in vivo*, there was no detectable proliferation of visceral epithelial cells, nor an increase in the proliferation of parietal epithelial cells in the rat [61] (Table 4). In agreement with this, no expression of PDGF receptor β-subunit, the receptor for the PDGF B-chain, could be demonstrated on visceral or parietal epithelial cells *in vivo* in the rat [22, 27]. An exception may be in human and primate kidneys, where the PDGF receptor β-subunit is present on parietal but, again, not on visceral glomerular epithelial cells [72].

Basic FGF has recently been shown to be mitogenic for glomerular epithelial cells in culture [73]. In contrast, when recombinant bFGF was injected or infused into normal rats there was no increase of glomerular epithelial cell proliferation [61]. However, to date it has not been determined whether previous injury of these cells, analogous to the experiments with mesangial cells (see above), renders them susceptible to a mitogenic effect of bFGF *in vivo* (Table 4).

Conclusions

Although marked progress has been made in the last 15 years in establishing glomerular cells in culture and in characterizing them, certain problems still have not been resolved. While glomerular cells are cultured under relatively similar conditions in many laboratories, no definitive consensus on optimal culture conditions exists, and this diversity as well as potential species differences have to be kept in mind when interpreting and, in particular, when comparing results that have been obtained with such cells. In the light of these caveats, it is not surprising that in some cases divergent results have been obtained, such as in the case of tumor necrosis factor synthesis by mesangial cells [74, 75] or their proliferative response after stimulation with interleukin 1 [59, 76–78] or interleukin 6 [59, 79, 80]. On the other hand, in particular when strong biological effects were observed, for example the mitogenicity of PDGF B-chain for mesangial cells, results between different laboratories show remarkable consistency.

In the previous sections we have outlined that not only cell culture data among themselves, but results obtained *in vitro* and *in vivo* may also yield both highly consistent as well as divergent data. However, in the cases where cell culture findings and findings in normal renal tissue differed, it has so far been possible in all instances to detect the respective *in vivo* correlate in disease states. Taken together the data currently available therefore suggest that under standard culture conditions glomerular cells *in vitro* exhibit the phenotype of a pathologically activated cell *in vivo*. The data also suggest that glomerular cell culture, at least when performed with non-immortalized cells, is not that unphysiological that it induces a complete dedifferentiation of the cells, since so far there are no examples of synthetic activities or response patterns *in vitro* that do not have any *in vivo* correlates.

Will glomerular cell culture eliminate the need for in vivo studies?

Glomerular cell culture has provided enormous insights into the capabilities of individual cell types. It has provided direct evidence for synthetic activities that can only, if at all, be inferred indirectly from observations *in vivo*, such as the production of extremely shortlived compounds including oxygen radicals or leucotrienes by glomerular cells [1–6, 81–83].

Certainly, the single most important role of glomerular cell culture resides in this qualitative description of the synthetic capabilities of these cells. However, while yielding a wealth of data, current cell culture has not allowed us to judge the relative importance of synthetic capabilities or response patterns in glomerular physiology and pathology. A good example of this is the synthesis of erythropoietin by mesangial cells in culture [84], which, however, *in vivo* is quite irrelevant compared to the renal interstitial production of erythropoietin [85]. Also, in cases where glomerular cells simultaneously produce mediators with antagonizing biological effects, for example vasodilatory prostaglandins and vasocontrictive endothelin [4–6, 21, 86], cell culture will hardly be able to provide clues to the *in vivo* net effect. Thus, at this point in time we believe that the clinical or physiological relevance of individual cell culture findings requires verification *in vivo*.

In an attempt to closer approach the *in vivo* situation many investigators have begun to extend or modify the "standard" cell culture techniques for glomerular cells, for example by investigating the effects of parallel stimulation with more than one mediator [59, 82, 87], by investigating the cells in serum-free culture [21, 59], by exposing them to stretch [88], by co-culturing different glomerular or glomerular and inflammatory cell types [89, 90], or by seeding the cells into a three-dimensional matrix [91–93]. On the other hand these studies too can only shed light on single aspects and furthermore are likely to introduce yet other specific problems inherent to these new techniques, such as the choice of the matrix when investigating cells in three-dimensional culture [91, 92]. Thus, only time will tell whether these newer techniques will provide further answers or yet more questions, and whether they at some point have the potential to eliminate the need for *in vivo* experiments.

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